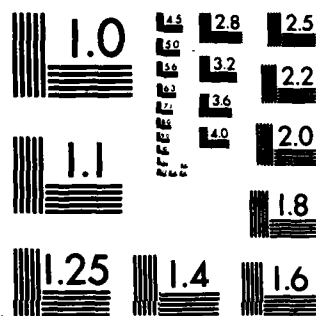END

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S-ESTERS OF THIOHYDOXIMIC ACID ESTERS--A NOVEL CLASS OF CHOLINESTERASE REACTIVATORS

Annual Report

by
Richard A. Kenley, Robert A. Howd, Carol W. Mosher,
and John S. Winterle

January 1981

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We have prepared a series of S-esters of thiohydroximic acid esters, given by the general formula $RC(:O)C(:NOH)S(CH_2)_nNR_2' \cdot HCl$, where $R = CH_3$; $CH_3OC_2H_4$, C_2H_5, $NO_2C_6H_4$; $n = 2,3'$, $R' = CH_3$, C_2H_5, or $i-C_3H_7$. The compounds were characterized with respect to acidity, nucleophilicity, and kinetics of reactivation of diisopropyl phosphoryl-acetylcholinesterase. The compounds were moderately active in reactivation of the enzyme, and activity is highly dependent on reactivator structure.

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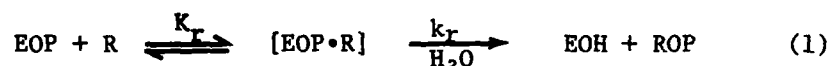
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SUMMARY

We have prepared a series of thiohydroximic acid S-esters and evaluated them in vitro with respect to their ability to reactivate diisopropyl phosphoryl-acetylcholinesterase. The compounds conform to the general formula: $RC(:O)C(:NOH)S(CH_2)_nNR_2' \cdot HCl$, where $R = CH_3$, C_6H_5, $4-CH_3OC_6H_4$, $4-NO_2C_6H_4$; $n = 2,3$; and $R' = CH_3$, C_2H_5, or $i-C_3H_7$. We also prepared $4-BrC_6H_4C(:NOH)S(CH_2)_2N(C_2H_5)_2 \cdot HCl$ and $4-CH_3OC_6H_4C(:O)C(:NOH)S(CH_2)_2N(C_2H_5)_2 \cdot CH_3Cl$ for comparison. The thiohydroximates exhibit oxime acid dissociation constants (pK_A) in the range 6.9 to 8.4, bracketing the value of $pK_a = 7.9$ believed to be optimum for acetylcholinesterase reactivation. The compounds are also good nucleophiles; bimolecular rate constants (k_n) for reaction with p-nitrophenyl acetate follow the expression $\log(k_n) = 6.7 - 0.69(14 - pK_A)$. The reactivation of diisopropyl phosphoryl-acetylcholinesterase is highly dependent on thiohydroximate structure: 4-h incubation of inhibited enzyme at $pH = 7.6$, 25°C with 1×10^{-3} M $CH_3OC_6H_4C(:O)C(:NOH)S(CH_2)_3N(CH_3)_2 \cdot HCl$, gives no detectable restoration of activity, whereas $4-CH_3OC_6H_4C(:O)C(:NOH)-S(CH_2)_2N(C_2H_5)_2 \cdot HCl$ restores inhibited enzyme activity to 58% of control under identical conditions. With thiohydroximate in excess over inhibited enzyme, the kinetics of reactivation are governed by a Michaelis constant (K_r) for binding thiohydroximate to the inhibited enzyme and a nucleophilic displacement rate constant (k_r) for attack on phosphorus.

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Introduction

A variety of toxic organophosphorus esters owe their biological activity to phosphylation[1] of a serine hydroxyl at the active site of acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7), AChE.[2] Inhibition of AChE leads to the accumulation of synaptic acetylcholine (ACh) and a concomitant disruption of nervous transmission.[3] Therapy for anti-AChE agent intoxication is based on coadministration of anticholinergics (e.g., atropine) to antagonize the effects of accumulated ACh and of AChe "re-activators".[4] These reactivators include nucleophilic species that displace inhibitor from the active surface and restore enzymatic activity.[5]

The last thirty years have witnessed considerable interest in elucidating the mechanism of AChE reactivation and it is recognized[6,7] that the reaction proceeds as shown in equation (1)

$$\text{EOP} + \text{R} \underset{}{\overset{K_r}{\rightleftharpoons}} [\text{EOP}\cdot\text{R}] \xrightarrow[\text{H}_2\text{O}]{k_r} \text{EOH} + \text{ROP} \qquad (1)$$

where: EOH is active enzyme, EOP is phosphylated enzyme, R is a reactivator, ROP is phosphylated reactivator, [EOP·R] is a complex between reactivator and inhibited enzyme, K_r (= [EOP][R]/[EOP·R]) is a Michaelis constant describing the affinity of the reactivator for the inhibited enzyme, and k_r is a rate constant for nucleophilic displacement of the inhibitor from the enzyme.

The development of useful reactivators has focused on compounds that combine a high affinity for the inhibited enzyme and strong nucleophilicity. In the later regard, compounds featuring the oximino (= NOH) functionality, have received the most attention since the oximate anion is a particularly

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strong nucleophile toward OP esters. The dual requirements for nucleophilicity and for dissociation of oxime into oximate anion at physiological pH combine to give an optimum value for the oxime acid dissociation constant (pKa), known empirically[5] to be $pK_a = 7.9$. Affinity for the inhibited enzyme has been approached by incorporating into reactivators cationic centers that provide for strong coulombic interaction with the anionic region(s) of AChE.

Although various approaches to the development of AChE reactivators have been taken, the pyridinium oximes, as a class, have been the compounds most studied. Specifically, 2-pyridinealdoxime methyl halides (2-PAM), 1,3-bis(4-hydroximinomethylpyridinium)propane dibromide (TMB-4), and 1,3-bis(4-hydroximinomethylpyridinium)dimethyl ether dichloride (toxogonin) are known to be powerful reactivators of phosphylated AChE as well as effective antidotes for OP ester intoxication.[8,11]

The proven therapeutic utility of pyridinium oximes notwithstanding, it is apparent that they have in common an important disadvantage in terms of limited tissue distribution. Hydrophobic cell membranes represent biological barriers to the transport of large ions from aqueous media into various tissues. This is especially true for passage of molecules from the serum into the central nervous system (CNS).[12-13] Nonionic OP esters are relatively lipophilic, and differ significantly from the hydrophilic pyridinium oximes in terms of tissue penetration. The OP esters pass rapidly into various tissues, including the CNS,[14-16] but the pyridinium oximes penetrate at a much slower rate. As a result, the pyridinum oximes exhibit a high degree of selectivity for activity at peripheral versus central sites.[17-27] Moreover the disproportionately high concentration

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of pyridinium oximes on the serum results in rapid renal excretion and
short biological half-lifes.[11,28,29]

The possibility that therapy of OP ester intoxication could be signi-
ficantly improved through the use of more lipophilic reactivators has
been appreciated by several investigators, and there have been various
attempts to design reactivators that better penetrate biological membranes.

One approach has been to capitalize on the inherent activity of 2-PAM
as a reactivator by modifying the general structure of the molecule. Thus
Wilson[30] and later Benschop et al[18] quaternized 2-pyridinealdoxime with
hydrophobic alkyl and aryl groups. Although these derivatives proved to
be lipophilic, they were too toxic to be of any practical value. The
tetrahydro-derivative of 2-PAM was prepared, but was devoid of activity
as a reactivator.[31] Higuchi and coworkers prepared 1,6-dihyro-2-PAM and
found that the compound penetrates the blood-brain barrier and functions
as a reactivator after in-vivo oxidation to 2-PAM.[32-35] However, several
studies have demonstrated that the conversion of 1,6-dihydro-2-PAM affords
no increase in antidotal efficacy relative to 2-PAM itself.[36-38]

There have also been several attempts to prepare nonquaternary AChE
reactivators based on novel molecular structures unrelated to the pyridinium
oximes. Examples include butanedioneoxime (DAM),[39] 4-dimethylaminobutanedione
2-oxime (isonitrosine),[18,40,41] 3-(diethylamino)propyl ester of oximinoacetic
acid (OA3),[18,42,43] 2-(diethylamino)ethyl S-ester of 4-bromobenzothiohydroximic
acid (LA54),[44-47] and 1,2,3-thiadiazole-5-aldoxime (TDA-5).[48] These non-
quaternary reactivators have demonstrated some limited advantages over the
pyridinium oximes, but to date none have been proven to be clearly superior
to 2-PAM in terms of overall therapeutic effectiveness.

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Although different factors limit the utility of the individual non-quaternary compounds described above, it is possible to rationalize their relative lack of antidotal efficacy primarily in terms of their poor activity as reactivators of inhibited AChE. In considering the molecular parameters that might affect the ability of the nonquaternary oximes to function as AChE reactivators, it is surprising to discover that structure-activity relationships for these materials have not been reported. In vitro screening of nonquaternary reactivators has been largely confined to determination of the percent activity restored to inhibited AChE after incubation with one concentration of a reactivator for a given time interval. While such determinations identify the relative potencies of structurally related materials, they provide no insight into the molecular requirements for activity.

Schoene[49] has used the determination of reactivation kinetics (the constants K_r and k_r) to good advantage in establishing structure-activity relationships for bis-pyridinium oximes. It seems that application of this technique to nonquaternary reactivators would be straightforward and highly useful with respect to design of novel compounds with enhanced reactivity.

In view of the foregoing, we have undertaken an investigation of a series of thiohydroximic acid esters, given by the formula:

$$RC\overset{O}{\underset{}{\|}}-C\overset{N\diagup^{OH}}{\underset{}{\|}}-S(CH_2)_n NR'_2 \cdot HCl$$
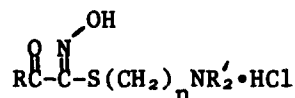
Our choice of this general framework was based on considerations of synthetic flexibility, structural similarity to AChE substrates (e.g., ACh and acetyl thiocholine), a functionality (protonated tertiary amine) providing for

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coulombic attraction to the anionic site of AChE, and the recognized requirement for compounds with oxime pK_a near 7.9. In the following we report the synthesis of the thiohydroximates and their characterization with respect to pK_a, nucleophilicity, and relative ability to reactivate diisopropyl phosphoryl-AChE. We also investigated the kinetics of reactivation of the inhibited enzyme with the objective of identifying structure-activity relationships for reactivation. For comparison we examined 2-PAM and $BrC_6H_4C(:NOH)SCH_2CH_2N(C_2H_5)_2 \cdot HCl$, a thiohydroximate that has been touted[45] as a particularly effective reactivator.

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Experimental Section

Melting points (uncorrected) were obtained with a Fischer-Johns apparatus, NMR spectra (in DMSO-d_6 solvent unless otherwise specified, Me_4Si internal reference, δ 0.0) were determined with a Varian model EM 390 or model XL-100-15 spectrometer; signals are designated as s (singlet), d (doublet), t (triplet), or m (multiplet). UV-visible spectra were measured with a Perkin-Elmer model 575 spectrophotometer. Enzyme kinetics were followed in 1-cm path length cuvettes using a Gilford model 20 spectrophotometer equipped with a four-position sample changer. The spectrophotometer output was coupled to a DEC MINC-11 computer programmed to give a continuously updated display of absorbance values and least squares slope, intercept and correlation coefficient for each cuvette. The system also provides for graphics display and permanent storage of the data in a disc file. A description of the program is available on request.* We conducted all kinetic experiments at 25°C and pH = 7.6 in 0.1 M MOPS buffer plus $MgCl_2$ (0.01 M), NaN_3 (0.002%), and bovine serum albumin (0.1%). Reported error limits for rate constants are standard deviations determined by least squares linear regression analysis.

Lyophilized electric eel AChE (Worthington) was used, with a nominal activity of 1.4×10^3 ACh units per milligram. Titration of the enzyme activity with ethyl p-nitrophenyl methylphosphonate indicated 6×10^{-9} mol of active sites per milligram of enzyme, in good agreement with the molecular weight of 2.3×10^5 reported[50] for the purified enzyme.

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*See appendix.

p-Nitrophenyl acetate (pNPA), 3-(N-morpholino)propanesulfunic acid (MOPS), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), and acetyl thiocholine were used as supplied by the manufacturer (Sigma Chemical Co.). We prepared isopropyl nitrite[51] in small amounts and stored it at 5°C until immediately before use. Where results of elemental analyses are given, only the symbols of the elements are indicated when experimental values agree within ±0.4% of theoretical.

Hydroximoyl Chlorides. The α-aroylhydroximoyl chlorides 4-RC$_6$H$_4$C(:O)C(:NOH)Cl, where R = H, CH$_3$O, or NO$_2$, were prepared by treating the corresponding acetophenones with i-C$_3$H$_7$ONO and HCl as described by Brachwitz.[52] CH$_3$C(:O)C(:NOH)Cl was similarly prepared from chloroacetone.[53] p-Bromobenzohydroximoyl chloride was prepared by chlorination of p-BrC$_6$H$_4$CHNOH.[54,55] Table I gives selected data for the hydroximoyl chlorides.

S-(N,N-Dialkylamino)alkyl Acyl- and Aroylthiohydroximates(SR 2458, 2462, 3008, 3010, 3014, and 3015). All compounds were prepared from the corresponding hydroximoyl chlorides by the same general procedure. SR 3015 and 3014, were isolated, respectively, as the methyl chloride and oxalic acid salts. The remaining compounds were isolated as the hydrochloride salts. Table II presents selected data for the thiohydroximates. The preparation of S-(2-diethylamino)ethyl 4-methoxybenzoylthiohydroximate hydrochloride (SR 2461) is described as a typical example.

Diethylaminoethanethiol hydrochloride (340 mg; 2 mmol was added to a solution of 404 mg (4 mmol) of triethylamine in 4 mL of CHCl$_3$. Then 427 mg (2 mmol) of α-chloro-α-oximino-p-methoxyacetophenone was added with stirring. The light orange-brown solution was stirred at room temperature for 4 h. Washing the solution with water removed little color; the solution

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Table I. Selected Data for Hydroximoyl Chlorides
RC(:NOH)Cl

R	Yield, %	Recryst. Solvent	mp °C	NMR (-NOH), δ
$C_6H_5C(:O)-$	52	$C_6H_6-CCl_4$	123–126	13.68
$4-NO_2C_6H_4C(:O)-$	18	C_6H_6	136–140	13.86
$4-CH_3OC_6H_4C(:O)-$	51	C_6H_6	131–133	13.40
$CH_3C(:O)-$	50	CCl_4	88–93	13.44
$4-BrC_6H_4-$	65	CCl_4	--	--

Table II. Selected Data for Thiohydroximates
RC(:NOH)S(CH$_2$)$_n$NR'·R"X

Compound	R	n	R'	R"X	Yield, %	TLC[a] R$_f$	mp, °C	NMR (-NOH), δ	UV[b] λ$_{max}$, nm	UV[b] ε, M^{-1} cm^{-1} × 10^{-4}	pK$_a$	Anal.
SR 2458	C$_6$H$_5$C(O)	2	C$_2$H$_5$	HCl	32	0.56	136–141	12.94	262	1.06	7.60	C,H,N,S,Cl
SR 2460	4-NO$_2$C$_6$H$_4$C(:O)	2	C$_2$H$_5$	HCl	18	0.57	170–172.5	13.39	269	1.74	7.1^f	C,H,N,S,Cl
SR 2461	4-CH$_3$OC$_6$H$_4$C(:O)	2	C$_2$H$_5$	HCl	65	0.65	139–140	12.69	230 303	1.12 1.50	7.8$_{:}$	C,H,N,S,Cl
SR 2462	CH$_3$C(:O)	2	C$_2$H$_5$	HCl	24	0.45	156–161	13.12	273	0.87	7.40	C,H,N,S,Cl
SR 3008	4-CH$_3$OC$_6$H$_4$C(:O)	2	CH$_3$	HCl	55	0.55	154–155.5	12.74	230 308	1.12 1.68	8.00	C,H,N,S,Cl
SR 3009	4-CH$_3$OC$_6$H$_4$C(:O)	2	(CH$_3$)$_2$CH	HCl	70	0.64	179–184	12.70	230 309	1.13 1.74	8.01	C,H,N,S,Cl
SR 3010	4-CH$_3$OC$_6$H$_4$C.(:O)	3	CH$_3$	HCl	d	0.30	c	12.50	d	d	d	d
SR 3014	4-CH$_3$OC$_6$H$_4$C(:O)	3	CH$_3$	[HOC(:O)]$_2$	29	0.42	151–153.5	e	d	d	8.42	C,H,N,S,Cl
SR 3015	4-CH$_3$OC$_6$H$_4$C(:O)	2	C$_2$H$_5$	CH$_3$I	48	0.39	c	f	d	d	7.82	C,H,N,S,Cl
LA54	4-BrC$_6$H$_4$	2	C$_2$H$_5$	HCl	41	0.63	146–149	12.16	233	1.41	9.28	C,H,N,S,Cl,Br

^{a}silica gel, CHCl$_3$ – MeOH (6:1).
^{b}In pH 7.6.0.13 M phosphate buffer.
^{c}Hygroscopic syrup.
^{d}Not determined.
^{e}Broad singlet, apparently including oxalate protons.
^{f}In CD$_3$OD solution, OH exchanged with OD.

was dried and concentrated to leave 780 mg of yellow syrup. An ether solution of the syrup was washed several times with water (which removed most of the color), dried, and concentrated. The syrupy residue (650 mg, showing several impurities by TLC) was purified on a silica gel column (14 x 2.5 cm) by "flash" chromatography[56] using $CHCH_3$-MeOH (8:1) as eluting solvent. From the fractions that showed no impurities, we obtained 479 mg of a light yellow syrup; TLC, R_f 0.6 ($CHCl_3$-MeOH, 6:1).

The syrup was dissolved in 10 mL of MeOH-ether (1:1), then filtered and treated with 0.5 mL of ether saturated with dry HCl. Ether was added to turbidity. Slight warming caused the separation of white crystals; after chilling, the product was collected, washed with ether, and dried at 25°C/0.1 mm to yield 415 mg (60%) of SR 2461. TLC ($CHCl_3$-MeOH, 6:1) R_f 0.65; NMR δ 12.69 (4, 1H, =NOH), 10.55 (broad, 1H, NH^+), 7.93 (d, 2H, Ar), 7.4 (d, 2H, Ar), 3.87 (s, 3H, OCH_3), 2.7-3.4 (m, 8H, CH_2), 1.13 (t, 6H, CH_3); IR (nujol) ν 2560br,m, 1661s, 1600s, 989s cm^{-1}. Slightly impure fractions of free base, 109 mg, were similarly converted to the hydrochloride and recrystallized from methanol-ether to yield 35 mg (5%), mp 134°-135°C.

S-(2-diethylamino)ethyl 4-Bromobenzothiohydroximate (LA54). 4-Bromo-benzohydroximoyl chloride (705 mg; 3.0 mmol) was treated with 2-diethyl-aminoethanethiol hydrochloride (510 mg; 3.0 mmol) and sodium methoxide (162 mg; 3.0 mmol) in 15 mL of isopropanol.[57] The crude product was purified by "flash" chromatography on silica gel ($CHCl_3$-MeOH, 6:1) and final recrystallization from methanol-ether to give 450 mg (41% yield) of white crystalline product, mp 146°-149°C. TLC ($CHCl_3$-MeOH, 6:1) R_f 0.63 (UV and I_2 detected); NMR δ 12.6 (s, 1H, NOH), 10.42 (broad, 1H, NH^+), 7.69 (φ, 4H, Ar), 3.3-2.8 (m, 8H, CH_2), 1.13 (t, 6H, CH_2); IR (nujol) ν 3215s, 2632s, 1587m, and 922s cm^{-1}.

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Acetylcholinesterase Assay. Two different spectrophotometric methods were used to determine AChE activity. The hydrolysis of PNPA to p-nitro-phenolate[58,59] (λ_{max} = 402 nm, ε = 1.50 x 10^4 M^{-1} cm^{-1})[60] was to assay enzyme activity in determinations of percent reactivation at 2, 4, and 24 h. For determination of the rate of dealkylation of diisopropyl phos-phoryl-AChE, the inhibition of AChE by reactivators, and kinetics of re-activation of diisopropyl phosphoryl-AChE, we assayed enzyme activity by the method of Ellman,[61] monitoring production of 5-thio-2-nitrobenzoic acid (λ_{max} = 412 nm, ε = 1.36 x 10^4 M^{-1} cm^{-1}). The pNPA assay proved to be the more convenient technique, but it was also less precise due to the relatively rapid spontaneous hydrolysis of pNPA. Details of the methods follows.

p-Nitrophenyl Acetate Method. The lyophilized enzyme is dissolved in buffer at a nominal concentration of 5 x 10^2 ACh units per mL and maintained at -10°C until immediately before use. p-NPA is dissolved in acetonitrile:ethanol (1:4) at 0.10 M. The enzyme stock solution is diluted by a factor of 1.0 x 10^3 and 10 µL of substrate stock solution is added to 990 µL of diluted enzyme to give final concentrations of 1.0 x 10^{-3} M substrate and 2 x 10^{-9} AChE. Under these conditions the rate of enzymatic hydrolysis of pNPA is $\approx$ 10 x 10^{-6} M min^{-1}, and the rate for spontaneous hydrolysis of pNPA is 2.3 x 10^{-6} M min^{-1}.

Ellman Method. The 500 ACh units per mL stock solution is diluted by a factor of 2.0 x 10^3 in MOPS buffer, DTNB is made to 1.0 x 10^{-2} M in pH 7, 0.1 M phosphate buffer, and acetyl thiocholine is made to 7.5 x 10^{-2} M in 1:9 H$_2$O:EtOH. Combination of 20 µL of enzyme solution plus 30 µL 0.1 M phosphate buffer, pH 8, gives an analytical solution with final concentrations

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of 2×10^{-11} M AChE, 3.0×10^{-4} M DTNB, and 7.5×10^{-4} M acetyl thiocholine. Under these conditions the enzyme-catalyzed rate of thiocholine production is $(8.75 \pm 0.01) \times 10^{-6}$ M min^{-1}, and the rate of spontaneous hydrolysis is 7.6×10^{-8} M min^{-1}.

Acetylcholinesterase Inhibition and Reactivation

p-Nitrophenyl Acetate Assay. A 100-µL aliquot of enzyme stock solution is diluted to 0.6 mL with MOPS buffer and 100 µL is removed and diluted 20-fold for noninhibited control assays. A 5-µL aliquot of 1.0×10^{-2} M DFP in ethanol is added to the remaining 0.5 mL of enzyme solution and incubated 3 min, during which period inhibition of the enzyme is complete. After the incubation period, the entire solution is added to a 1 cm x 20 cm glass column packed to a bed height of 12 cm with Sephadex G-50, 50-100 mesh, the solution is eluted under suction with MOPS buffer, and 1.0 to 2.0-mL fractions are collected. The enzyme elutes primarily in the 2- to 4-mL fraction and DFP primarily in 7- to 9-mL fraction. The elution of DFP in the AChE fraction was negligible as evidenced by incubating noninhibited AChE with the 2- to 4-mL fraction and assaying for enzymatic activity. For reactivation studies candidate reactivators are dissolved to 1.0×10^{-2} M in water and diluted to 3.0×10^{-6} to 2.0×10^{-3} M in 100 µL of inhibited enzyme solution plus the quantity of buffer required to give 200 µL final volume. These solutions are incubated at 25°C±0.2°C in a shaker bath, and 25-µL aliquots are removed at timed intervals. The 25-µL aliquots are added to 965-µL MOPS buffer plus 10 µL of 1.0×10^{-2} M pNPA, and the enzyme activity is assayed. Thus the final concentrations in the assay solutions are as follows: 1×10^{-9} M inhibited AChE, 3.00×10^{-8} to 2.00×10^{-5} M reactivator, and 1.0×10^{-3} M substrate. Observed enzyme

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activities are corrected (see Results Section) for spontaneous and re-activator-catalyzed hydrolysis of substrate to give net activities. 100% enzyme activity is defined as the activity restored by incubation of inhibited enzyme with 1.0×10^{-3} M 2-PAM.

Ellman Procedure. The general procedure described above is followed except that lower enzyme concentrations are required. Typically a 10-μL aliquot of stock AChE is diluted to 0.6 mL in MOPS buffer and incubated with a 4-μL aliquot of 1.0×10^{-2} M DFP for 10 min. From the 1- to 4-ml column fraction of inhibited enzyme, 400-μL aliquots are withdrawn and diluted to 600-μL with MOPS buffer and the volume of reactivator stock solution required to give 3.00×10^{-6} to 2.00×10^{-3} M reactivator concentration. At timed intervals, 20-μL aliquots of the enzyme plus re-activator solution are withdrawn and assayed as described above. Thus the final concentrations in the assay solution are as follows: 1.0×10^{-10} M AChE, 3.00×10^{-8} to 2.00×10^{-5} M reactivator, 3.0×10^{-4} M DTNB, and 7.5×10^{-4} M acetyl thiocholine.

Results and Discussion

Synthesis, Structure, and Acidity. We prepared the thiohydroximates
described in Table II by reaction (2) and (3)

$$RCCH_3 + iPrONO \xrightarrow{HCl} R-C-C-Cl \qquad (2)$$

$$R-C-C-Cl + HS(CH_2)_n NR'_2 \xrightarrow{Et_3N} R-C-C-S(CH_2)_n NR'_2 \quad (3)$$
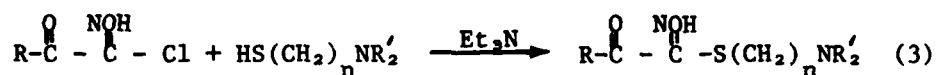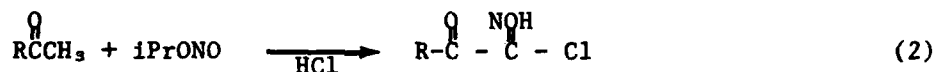
Unoptimized yields of reaction (2) ranged from 18% to 70%, based on the
amount of hydroxamoyl chloride used.

Oxime acid dissociation constants (pKa) were measured by spectrophoto-
metric determination of oximate concentration in buffers of various pH.[62]
From the data in Table II for the SR-series compounds, a plot (not shown)
of pKa versus oxime proton NMR chemical shift is linear and conforms to
equation (4)

$$pKa = (25.3 \pm 2.1) - (1.36 \pm 0.16)\,\delta \qquad (4)$$

For the aroylthiohydroximates (SR 2458, 2460, and 2461) a plot (not shown)
of pKa versus Hammet substituent constant (σ_p)[63] is also linear and conforms
to equation (5)

$$pKa = (7.63 \pm 0.02) - 0.63 \pm 0.05)\,\sigma_p \qquad (5)$$

These correlations provide an indication of the validity of the pKa values,
an important consideration because we have related thiohydroximate reactivity
to the concentration of the dissociated form at a given pH and hence to

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the acidity of the oxime functionality. We determined pKa = 7.99 for
2-PAM, which is in good agreement with the value of 7.92 reported by
Schoene and Strake.[49] Our value of pKa = 9.28 for LA54 is lower than that
(pKa = 10.42) determined potentiometrically by Krivenchuk et al.[45] We
prefer our value because the spectrophotometric technique should be less
sensitive than the potentiometric method to interference from the tertiary
amine acid-base equilibrium.

We have considered the possibility of syn and anti isomerization in
the thiohydroximates insofar as the configuration of the oxime functionality
can have a profound influence on the reactivation of inhibited AChE.[10]
Exner and coworkers find that benzohydroxamoyl chlorides[64] and methyl
benzothiohydroximate[65] adopt the syn configuration solution.

syn-benzohydroxamoyl
chlorides

syn-methyl
benzothiohydroximate

Line Drawing 1

16
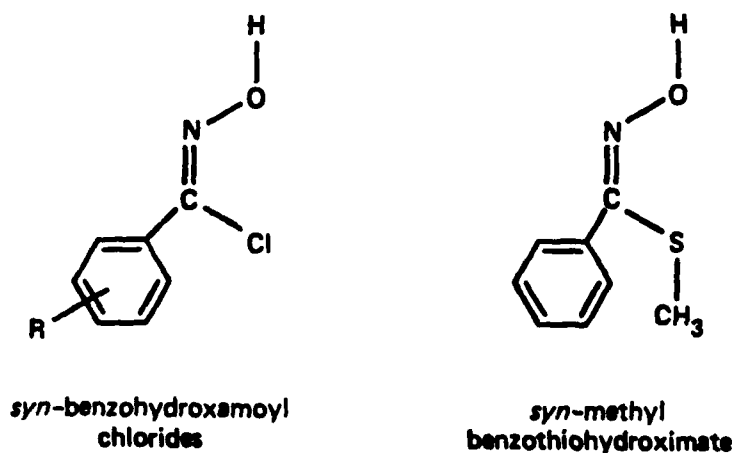
These workers also report IR O-H stretching frequencies near 3560 cm^{-1}, for these compounds in CHCl$_3$. We find that LA54 and the hydroxamoyl chlorides listed in Table I exhibit O-H stretching frequencies at 3200 to 3300 cm^{-1} for spectra taken in nujol mulls. By analogy, a _syn_ configuration seems likely for these compounds. Contrasting this, the SR-series thiohydroximates nujol mull spectra show no sharp O-H bands in the 3000 to 4000 cm^{-1} region. An IR spectrum of the free base of SR 2458 in CHCl$_3$ solution reveals a very broad band centered at 2564 cm^{-1} that it typical of hydrogen-bonded O-H.[66] These observations suggest the _anti_ configuration for the SR-series thio-hydroximates, with six-center intramolecular hydrogen bonding of oximino proton to the α-carbonyl group.
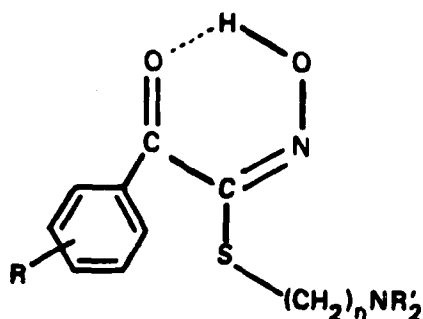
anti-aroylthiohydroximate

Line Drawing 2

Nucleophilicity. As a measure of the inherent reactivity of the thio-hydroximates, we determined values for the bimolecular rate constant, k_n, for reaction of the thiohydroximates with pNPA. We measured initial rates of production of p-nitrophenolate (pNP) from the reaction of 1.0×10^{-3} M

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pNPA with 10.0 to 100 x 10^{-6} M thiohydroximate at pH 7.6 and 25°C. Under these conditions the rate of pNP formation is zero-order, consistent with the rate law given in equation (6):

$$+ d[pNP]/dt = k_n[pNPA][OX] + k_o[pNPA] \qquad (6)$$

where [OX] is the concentration of the anionic form of the thiohydroximate, and k_o is the pseudo-first-order rate constant for spontaneous hydrolysis of [pNPA]. At low conversions both [pNPA] and [OX] remain constant and equation (7) reduces to:

$$k_{eQ} = +d[pNP]/dt \cdot [pNPA]^{-1} = k_n[OX] + k_o \qquad (8)$$

where k_{eQ} is the equivalent first-order rate constant for pNP production, and [OX] is calculated from the concentration of added test compound [HOX] and equation (9).

$$[OX] = [HOX] \cdot \{1 + antilog \ (pK_A - 7.6)\}^{-1} \qquad (9)$$

In accordance with equation (8), plots (not shown) of k_{eQ} versus [OX] for the SR-series thiohydroximates, 2-PAM, and LA54 are linear with slope = k_n and intercept = k_o. Table III gives values of k_n and k_o so determined, along with values for pK_B (=14 - pK_a), the conjugate base ionization constant for the test compounds.

As required by equation (9), the k_o values are essentially independent of the reactivator used. The average for all values of k_o from Table III is (2.3 ±0.4) x 10^{-3} min^{-1}, which is in good agreement with the value of k_o = 2.1 x 10^{-3} min^{-1} reported by Jencks and Gilchrist.[67] For 2-PAM, the value of k_n = (9.6 ±0.8) x 10^2 M^{-1} compares to the value k_n = 4.0 x 10^2 M^{-1} min^{-1}

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Table III. Rate Constants for Hydrolysis of p-Nitrophenyl Acetate (pNPA) in the Presence of Thiohydroximates[a]

Compound[b]	k_n:[c] M^{-1} min^{-1} x 10^{-2}	k_o:[d] Min^{-1} x 10^3	pK_B[e]
SR 2458	3.9 ± 0.2	2.0 ± 0.4	6.40
SR 2460	1.4 ± 0.1	2.8 ± 0.4	6.86
SR 2461	6.1 ± 0.02	2.0 ± 0.4	6.18
SR 2462	2.7 ± 0.1	2.8 ± 0.6	6.60
SR 3008	5.1 ± 0.6	1.7 ± 0.4	6.00
SR 3009	5.6 ± 0.4	2.4 ± 0.3	5.99
SR 3014	13.4 ± 0.05	2.2 ± 0.2	5.58
SR 3015	3.9 ± 0.03	1.8 ± 0.3	6.18
LA54	2.4 ± 0.2	2.6 ± 0.3	4.72
2PAM	9.6 ± 0.8	2.9 ± 1.2	6.01

[a]Determined from equivalent first-order rates, k_{eq}, for p-nitro-phenolate production from 1.0 x 10^{-3} M pNPA in the presence of 10 to 100 x 10^{-6} M test compound at pH 7.6 and 25°C in 0.1 M MOPS buffer; see equation (7).

[b]See Table II.

[c]Nucleophilic displacement rate constant from slope of plot of (OX) vs k_{eq}; see equations (8) and (9).

[d]Spontaneous hydrolysis rate constant, from intercept of plot of [OX] vs k_{eq}; see equations (8) and (9).

[e]pK_B = 14 - pK_A.

determined by Bergmann and Govrin[68] at pH = 8.0 and 25°C.

Figure 1 is a Bronsted plot (log k_n versus pK_B) of the data of Table III. The data for the SR-series compounds conform to equation (10):

$$\log (k_n) = (6.9 \pm 0.5) = (0.69 \pm 0.08)\ pK_B \qquad (10)$$

where the β-value (slope) of 0.69 ±0.08 compares to the value of β = 0.8 commonly observed for reaction of pNPA with oxygen nucleophiles.[67,69]

Thus the thiohydroximates behave as nucleophiles in the anticipated manner. Because the nucleophilicity is proportional to acidity of the oxime functionality and because the acidities of the aroylthiohydroximates obey a Hammet linear free energy relationship, it is possible to "fine tune" both acidity and nucleophilicity by appropriate selection of aromatic substituent groups.

AChE Reactivation Control Experiments. All observed enzyme activities were corrected for spontaneous and oximate-catalyzed hydrolysis of substrate. For pNPA as substrate (see Experimental Methods Section:, the initial rates of oximate-catalyzed hydrolysis were actually greater than the enzyme-catalyzed rates for high reactivator concentrations. However, for the high concentration (1.0×10^{-3}) of pNPA used in the assay, the text compounds (10.0 to 100 $\times 10^{-6}$ M in the assay) were rapidly consumed. Thus after an initially high rate of pNP production, the rate leveled off within 2 to 4 min to a value dependent only on the enzyme-catalyzed reaction plus a contribution due to spontaneous hydrolysis. The latter could be accounted for by the value of k_o determined above. In the case of reactions assayed by the Ellman technique, corrections for nonenzymatic substrate hydrolysis were smaller, and could be made by running blanks with reactivator in an

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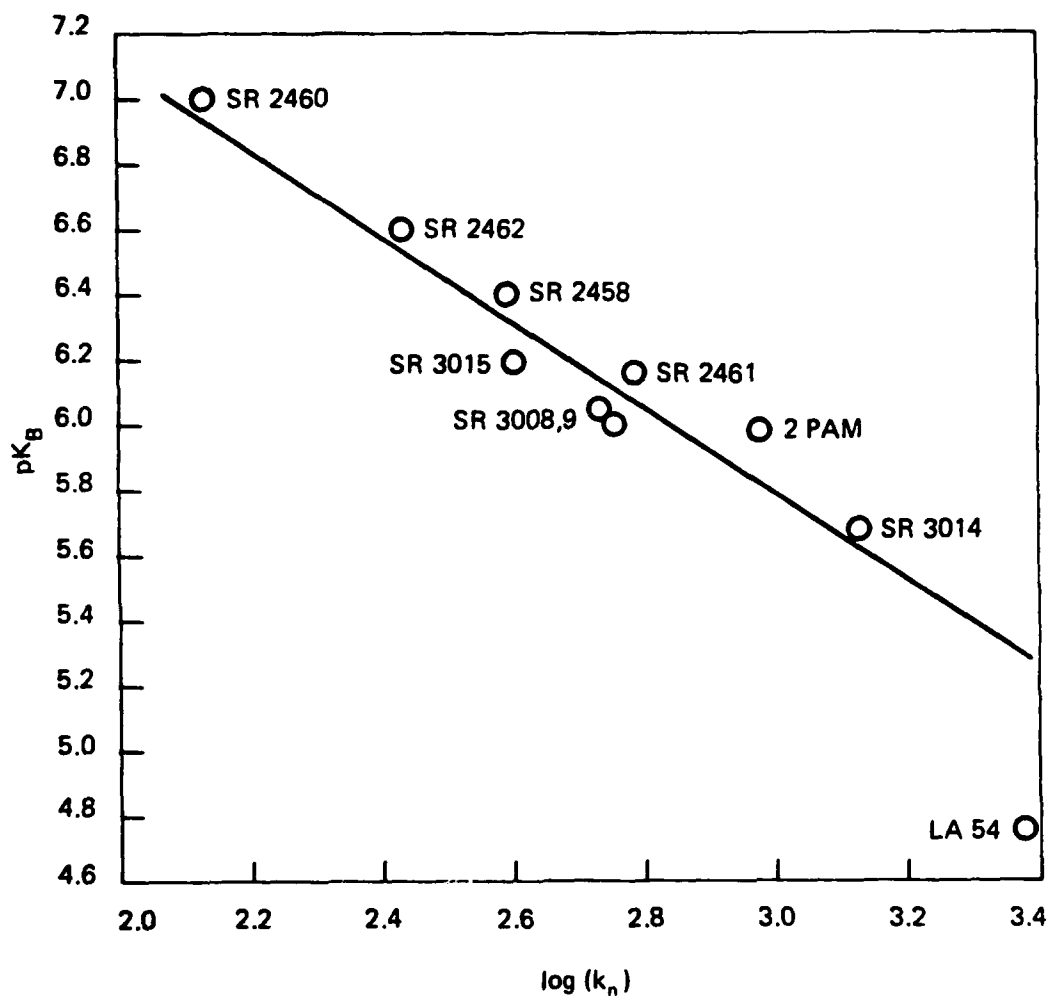
FIGURE 1   CONJUGATE BASE IONIZATION CONSTANTS (pK_B) VERSUS LOGARITHM
OF BIMOLECULAR NUCLEOPHILIC DISPLACEMENT RATE CONSTANT (k_n)
FOR HYDROLYSIS OF p-NITROPHENYL ACETATE IN THE PRESENCE OF
VARIOUS REACTIVATORS AT pH = 7.6, 25°C

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appropriate concentration, but with no enzyme added.

Spontaneous reactivation of diisopropyl phosphoryl-AChE was negligible over 24 h. However, the inhibited enzyme (EOP) rapidly converted ("aged") into a nonreactivatable species (EOP') as shown in reaction (11):

$$EOP \xrightarrow{\quad k_a \quad} EOP' \qquad (11)$$

where EOP is diisopropyl phosphoryl-AChE and EOP' is a dealkylated-, inhibited-enzyme in accordance with the accepted[5] mechanism for this phenomenon. We determined k_a under our experimental conditions by incubating the inhibited enzyme for time intervals before reactivating with 1.0×10^{-3} M 2-PAM. Semilog plots of A_t/A_o versus time (where A_t is AChE activity after incubation for t min before reactivation with 2-PAM, and A_o is activity of inhibited enzyme reactivated immediately) were linear for at least three half-lives. For three determinations the average value of k_a was $(1.61\pm0.08) \times 10^{-3}$ min^{-1}, corresponding to a half-life for aging of 7.1 h.

We incubated AChE with the various test compounds to check for reversible or irreversible inhibition of the enzyme under the conditions of the reactivation experiments. Thus, we incubated AChE with each test compound $(1.00 \times 10^{-3}$ M) and withdrew aliquots at 0, 3, and 24 h for determination of enzyme activity. A sample of AChE without added test compound served as control. Except for SR 3009, none of the compounds gave any evidence of inhibition of the enzyme using this procedure. For SR 3009 the average value of enzyme activity at 0, 3 and 24 h was 92±12% of control. For all the other compounds at all three time points, the average value of the enzyme activity was 102±3% of control. Thus the inhibition of AChE due

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to the reactivators themselves can be neglected in our assays of enzyme activity.

Finally we checked the thiohydroximates for hydrolytic stability at pH = 7.6 and 25°C. As evidenced by UV-visible spectra and by rate constants, k_n, for reaction with pNPA, hydrolysis of the compounds over 24 h was negligible.

<u>Percent AChE Reactivation</u>. To determine the relative activities of the thiohydroximates as reactivators of diisopropyl phosphoryl-AChE, we added the DFP-inhibited enzyme to 1.00 x 10^{-3} M solution of the test compounds and determined enzyme activity after 2-, 4-, and 24-h incubation periods. Percent reactivation is calculated according to equation (12):

$$\%r_t = 100 \ (1 - E_t/E_o) \tag{12}$$

where $\%r_t$ is the percent reactivation after t min incubation, E_t is the enzyme activity at time t, and E_o is the control enzyme activity. Table IV summarizes the data for the SR-series thiohydroximates, plus 2-PAM and LA54.

From the table, it is seen that the SR-series thiohydroximates do function as reactivators and that their activity is highly dependent on structure. In the series of dimethyl, diethyl, and diisopropylaminoethyl S-esters of p-methoxybenzoylthiohydroximic acid (SR 3008, 3009, and 2461), activity decreases with increasing size of the alkyl substituent, indicating a steric effect on the reactivation. It is also significant that the quaternary thiohydroximate SR 3015 is actually less reactive than the tertiary analog SR 2461. We anticipated that the tertiary alkylamine functionality would be completely protonated at pH = 7.6 and that the

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Table IV. Percent Reactivation of ($\%r_t$) of Diisopropyl
Phosphoryl-AChE after Incubation with 1.00×10^{-3} M
Reactivator at 25°C, pH = 7.6

Compound[a]	$\%r_t$[b] After Incubation for t hr		
	t = 2	t = 4	t = 24
SR 2458[c]	63	83	80
SR 2460	18	20	25
SR 2461	31	58	55
SR 2462	13	28	23
SR 3008	56	65	71
SR 3009	4.3	2.8	21
SR 3014	0.0	0.0	16
SR 3015	5.6	9.5	18
LA54[c]	8	8	9
2PAM	109	105	85

[a]See Table II.

[b]$\%r_t$ calculated from equation (12).

[c]Reactivator concentration = 2.00×10^{-3} M.

protonated species would provide the coulombic interaction with the anionic
subsite necessary for enhanced affinity of the reactivator for the inhibited
enzyme. If this were not the case, the quaternary ammonium analog would
predictably exhibit considerably higher activity than the nonquaternary
reactivator. The fact that SR 2461 is actually more reactive than the
methylated SR 3009 can be rationalized on the basis of strong coulombic
interactions for both compounds plus a steric effect that lowers the affinity
of the N-methyl derivative for the inhibited enzyme.

Comparison of the 2-(dimethylamino)ethyl and 3-(dimethylamino)propyl
S-esters of p-methoxybenzoylthiohydroximic acid (SR 3008, and 3014, respectively)
shows lower activity for the 3-(dimethylamino)propyl analog. While this
could be interpreted as an effect of reactivator structure on activity,
it must be noted that the pKa of SR 3014 (Table II) is 0.6 log units higher
than that of SR 2461. Therefore at pH 7.6, SR 2461 is 40% ionized [equation
(9)], whereas SR 3014 is only 14% ionized to the nucleophilic oximate anion.
This example demonstrates that the simple screening for activity by deter-
mining percent reactivation under standard conditions suffices to rate
similar compounds in terms of relative potency, but can fail to permit an
elucidation of the molecular parameters that govern reactivity.

The shortcomings of the method are also apparent when 2-PAM is compared
with the thiohydroximates. Because reactivation by 2-PAM is complete within
2 h, it is impossible to use the data of Table IV to quantitatively compare
it with the thiohydroximates. To do so requires a careful study of re-
activation as a function of time and reactivator concentration. Results
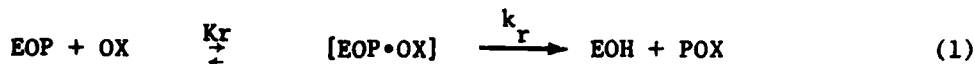of such a study are presented in the section that follows.

Finally, the data for LA54 deserve comment. We find LA54 to be con-
siderably inferior to 2-PAM as a reactivator of diisopropyl phosphoryl-

AChE and attribute the lack of activity of LA54 primarily to its high

pK$_a$ (= 9.42) and the correspondingly low fraction (1.4% at pH = 7.6) of

the ionized species available to reactivate the enzyme. These results

are in marked contrast to those of Krivenchuk et al.[45] who obtained 35%

and 41% reactivation of ethyl methylphosphonylbutyrylcholinesterase after

incubation with 2-PAM and LA54, respectively.

Even considering the differences in inhibited enzymes used, it is

difficult to rationalize the large differences in the relative activities

of LA54 and 2-PAM reported by us and by Krivenchuk and coworkers. In the

absence of additional details · experimental method employed

by Krivenchuk et al., we canno· ut other reasons for this

apparent discrepancy. In any roves to be a very poor reactivator

in our experiments, and we believe that published reports of its antidotal

effectiveness[44-47] should be interpreted with some caution.

AChE Reactivation Kinetics. The work of several authors[7,49] makes

it clear that the kinetics of reactivation of phosphylated AChE cannot be

adequately described by a simple bimolecular substitution reaction mechanism.

Rather, the mechanism involves reversible formation of a Michaelis complex

between reactivator and inhibited enzyme followed by a nucleophilic dis-

placement reaction on phosphorus, as shown in equation (1). For the

particular case of reactivation of diisopropyl phosphoryl-AChE, dealkylation

of the inhibited enzyme [reaction (11)] is sufficiently rapid that this

reaction must also be taken into account in determining reactivation kinetics.

De Jong and Wolring[70,71] have devised a kinetic scheme that allows

for parallel processes of reactivation and dealkylation. The scheme

consists of reactions (1), (11), and (13):

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$$\text{EOP} + \text{OX} \quad \overset{K_r}{\underset{}{\rightleftharpoons}} \quad [\text{EOP·OX}] \quad \xrightarrow{k_r} \quad \text{EOH} + \text{POX} \tag{1}$$

$$\text{EOP} \quad \xrightarrow{k_a} \quad \text{EOP}' \tag{11}$$

$$[\text{EOP·OX}] \quad \xrightarrow{k_a'} \quad \text{EOP}' \tag{13}$$

where the reactivator is represented by the oximate anion, OX, and K_r, k_r, and k_a are as previously defined. Provision is made in reaction (13) for dealkylation of the Michaelis complex, [EOP·OX], to the nonreactivatable form of the inhibited enzyme, EOP', with a rate constant, k_a', that may or may not differ from k_a.

Following the derivation of De Jong and Wolring, it can be shown that:

$$\ln(\%r_\infty - \%r_t) = \ln(\%r_\infty) - k_{obs} \cdot t \tag{14}$$

$$k_{obs} - k_a = \frac{k_{max}[\text{OX}]}{K_r + [\text{OX}]} \tag{15}$$

$$k_{max} = k_a' - k_a + k_r \tag{16}$$

$$k_a' = \{(k_{max} + k_a)[\text{EOP}']_\infty / [\text{EOH}]_\infty - k_a k_r [\text{OX}]^{-1}\}(1 + [\text{EOP}']_\infty / [\text{EOP}]_\infty)^{-1} \tag{17}$$

$$[\text{EOP}']_\infty / [\text{EOH}]_\infty = (100 - \%r_\infty)/\%r_\infty \tag{18}$$

where k_{obs} is the pseudo-first-order (oximate in excess) rate constant for reactivation; $\%r_\infty$ is the maximum attainable percent reactivation; k_{max} is defined as in equation (16); $[\text{EOP}']_\infty$ is the amount of nonreactivatable enzyme at infinite time; $[\text{EOH}]_\infty$ is the concentration of active enzyme at infinite time; and k_a is determined in an independent experiment according to equation (11).

Rearranging equation (17) gives

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$$\frac{[EOP']_{\infty}}{[EOH]_{\infty}} = \frac{k_a k_r [OX]^{-1}}{k_r} + \frac{k_a'}{k_r} \tag{19}$$

Similarly, rearranging equation (15) gives

$$(k_{obs} - k_a)^{-1} = \frac{K_r [OX]^{-1}}{k_{max}} + \frac{1}{k_{max}} \tag{20}$$

Thus a plot of $\ln(\%r_{\infty} - \%r_t)$ versus time gives slope $= -k_{obs}$ and intercept $=$ $\ln(\%r_{\infty})$; a plot of $[EOP']_{\infty}/[EOH]_{\infty}$ versus $[OX]^{-1}$ gives slope $= k_a K_r/k_r$, and intercept $= k_a'/k_r$; and a plot of $(k_{obs} - k_a)^{-1}$ versus $[OX^{-1}]$ gives slope $= K_r/k_{max}$ and intercept $= 1/k_{max}$. Because k_a is independently known (vide supra), the determination of $\%r_{\infty}$ and $\%r_t$ as a function of time and reactivator concentration permits calculation of the important kinetic constants K_r and k_r. Table V summarizes the necessary derivations. Wang and Braid[7c] have shown that at low reactivator concentration, equation (21) holds:

$$k_b = k_r/K_r \tag{21}$$

where k_b is a bimolecular rate constant for reactivation in the limit of low reactivator concentration. Finally, we define k_{eff} as a quantitative measure of the effectiveness of reactivation. This term is governed by the intrinsic activity of an oximate anion (k_b) and the fraction of re-activator that exists in the dissociated form at pH = 7.6. Thus from equations (9) and (21), k_{eff} is given by equation (22):

$$k_{eff} = k_b [1 + antilog (pKa - 7.6]^{-1} \tag{22}$$

To probe these relationships, we determined $\%r_{\infty}$ and $\%r_t$ as a function

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Table V. Formulas for Calculating Kinetic Constants for
Reactivation of Inhibited AChE

| Plot	Abscissa	Ordinate	Slope(n)	Intercept(n)	Calculate	Equation
1	t	$\ln(\%r_\infty - \%r_t)$	$S_1 = -k_{obs}$	$I_1 = \ln(\%r_{oo})$	$[EOP']/[EOH] = (100 - \exp[I_1])/\exp[I_1]$	(14), (18)
2	$[OX]^{-1}$	$[EOP']_\infty /[EOH]_\infty^{-1}$	$S_2 = k_a K_r/k_r$	$I_2 = k_a'/k_r$	$k_a' = I_2 k_r$	(19)
3	$[OX]^{-1}$	$(k_{obs} - k_a)^{-1}$	$S_3 = K_r/k_{max}$	$I_3 = 1/k_{max}$	$K_r = S_3/I_3$ $k_r = [(I_2)^{-1} + k_a]\cdot$ $[1 + I_1]^{-1}$	(16), (20) (20), (19)

of time for various concentrations of 2-PAM, SR 2458, 2461, and 3008.
Figure 2 is a sample plot of equation (14) for 1.00×10^{-3} M 2-PAM, and
SR 2461, and 0.200×10^{-3} M SR 3008. The linearity of the plots indicates
that complicating factors, such as reinhibition of the enzyme by phos-
phorylated reactivator, are unimportant under the experimental conditions.[71]
Table VI summarizes the values of k_{obs} and $\ln(\%r_{\infty})$ obtained for a range
of concentrations of all four reactivators. Figure 3 is a double reciprocal
plot of $(k_{obs} - k_a)^{-1}$ versus $[OX]^{-1}$ for SR 2458, 2461, and 3008. The plot
for 2-PAM is not shown because of the difference in scale for $[OX]^{-1}$, but
the data for 2-PAM conform reasonably well to equation (20). A similar
plot (not shown) of $[OX]^{-1}$ versus $[EOP']^{-1}$ versus $[EOP']_{\infty}/[EOH]_{\infty}$ conforms
to equation (19) for the reactivators investigated. Data from Table VI
and from least squares treatment of the data according to equations (19)
and (20) are summarized in Table VII along with kinetic constants calculated
as in Table V and equations (21) and (22).

At this point, some discussion of the precision of the measurements
seems appropriate. Accurate determination of $\%r_{\infty}$ is essential for
reliable calculation of values for k_{obs} and especially for the ratio
$[EOP']_{\infty}/[EOH]_{\infty}$. We did not make replicate determinations of $\%r_{\infty}$ values
in our experiments, and this is a major source of uncertainty in calculating
kinetic constants. The uncertainty is largest for least squares deter-
minations of intercepts calculated from equation (19). These intercepts
are used to calculate k_a', and we view the values for k_a' in Table VII as
tentative. The intercepts calculated from equation (19) are also subject
to a degree of uncertainty, and this must be taken into consideration
when interpreting values for k_r.

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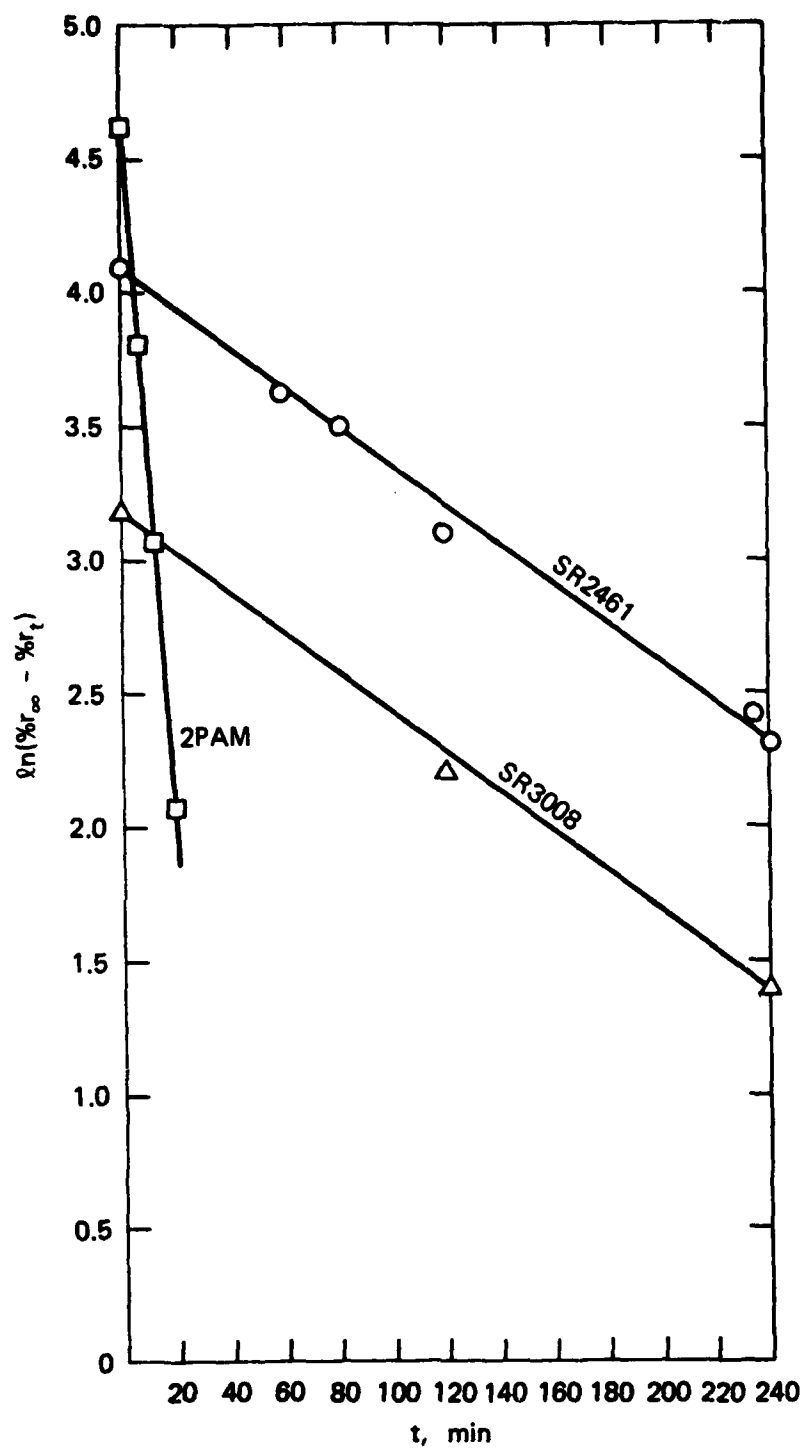
FIGURE 2   NATURAL LOGARITHM OF PERCENT MAXIMUM REACTIVATION MINUS
REACTIVATION AT TIME t, ℓn(%r∞ - %rₜ) VERSUS TIME AT 25°C, pH = 7.6
FOR REACTIVATION OF DIISOPROPYL PHOSPHORYL-AChE WITH
1.0×10^{-3}M 2PAM (□—□), 1.0×10^{-3}M SR2461 (O —— O), AND
0.20×10^{-3}M SR3008 (△ —— △)

31
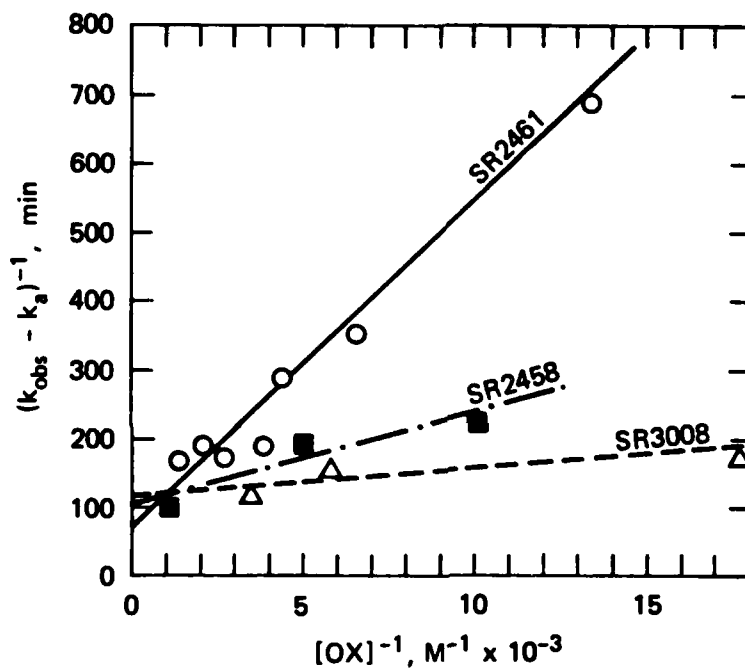
FIGURE 3 DOUBLE RECIPROCAL PLOT OF OBSERVED FIRST-ORDER REACTIVATION
RATE CONSTANT MINUS DEALKYLATION RATE CONSTANT, $(k_{obs} - k_a)^{-1}$
VERSUS OXIMATE CONCENTRATION ($[OX]^{-1}$) FOR REACTIVATION OF
DIISOPROPYL PHOSPHORYL-AChE BY SR2458 (■ — ·— ■), SR2461 (O —— O),
AND SR3008 (△ - - -△)

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Table VI. Pseudo-First-Order Observed Rate Constants, k_{obs}, for Reactivation of Diisopropyl Phosphoryl-AChE as a Function of Reactivator Concentration at 25°C, pH = 7.6

Compound[a]	[HOX], M x 10^4	[OX],[b] M x 10^4	k_{obs},[c] min^{-1} x 10^3	$\ln(\%r_\infty)$[d]
SR 2458	2.00	1.00	5.90 ± 0.8	3.19 ± 0.04
	4.00	2.00	6.82 ± 1.7	3.39 ± 0.10
	20.0	10.0	11.0[e]	4.42
SR 2461	2.00	0.752	3.02 ± 0.16	3.30 ± 0.01
	4.00	1.52	4.40 ± 0.62	3.49 ± 0.04
	6.00	2.26	5.13 ± 1.0	3.79 ± 0.16
	7.00	2.63	7.12 ± 0.27	4.10 ± 0.03
	10.0	3.76	7.24 ± 0.26	4.05 ± 0.04
	12.0	4.51	6.90 ± 0.17	4.22 ± 0.02
	20.0	7.52	7.65 ± 0.47	4.30 ± 0.06
SR 3008	2.00	0.569	7.46 ± 0.41	3.15 ± 0.063
	6.00	1.71	8.17 ± 1.1	3.83 ± 0.16
	10.0	2.85	10.3 ± 1.5	4.15 ± 0.23
2PAM	0.0300	0.00870	6.57 ± 0.38	3.93 ± 0.049
	0.0800	0.0232	9.31 ± 0.11	4.20 ± 0.014
	0.100	0.0289	19.6 ± 0.80	f
	0.160	0.0463	20.2 ± 1.1	4.42 ± 0.083
	0.200	0.0571	29.8 ± 1.4	f
	10.0	2.89	125 ± 10	4.49 ± 0.12

[a] See Table II.

[b] Calculated from [HOX] and equation (9).

[c] Calculated from slope of least squares plot of data according to equation (14).

[d] Calculated from intercept of least squares plot of data according to equation (14).

[e] Calculated from three points, including $\%r_\infty$, no valid statistical data.

[f] No infinity point measured. Slopes calculated from initial points by the method of Guggenheim (ref. 72).

Table VII. Kinetic Constants for Reactivation of Diisopropyl Phosphoryl-AChE at 25°C, pH = 7.6

Compound[a]	[InX] M x 10^3	[OX]^-1 [b] M^-1 x 10^-2	$(k_{obs}-k_a)^{-1}$ [c] min	[EOP]'[d] /[EOX]	k_a'/k_r [e]	$\dfrac{k_a K_r}{k_r}$ [f] M x 10^5	K_r/k_{max} [g] M·min x 10^3	$1/k_{max}$ [h] min	k_a' [i] x 10^3 min^-1	k_r' [j] x 10^2 min^-1	K_r [k] M x 10^5	k_b [l] M^-1 min^-1	k_{eff} [m] M^-1 min^-1	k_{eff} (Relative)
SR 2458	2.00	10.0	231	3.12	0.21 ± 0.7	3.2 ± 1.1	1.4 ± 0.36	103 ± 24	2.0	9.3	1.3	70	35	4.5
	4.00	5.00	190	2.37										
	20.0	1.00	106	0.203										
SR 2461	2.00	13.3	689	2.69	0.17 ± 0.18	2.1 ± 0.30	4.6 ± 0.35	66 ± 22	2.4	14	6.9	21	7.8	1
	4.00	6.65	353	2.05										
	6.00	4.42	281	1.26										
	7.00	3.80	180	0.742										
	10.0	2.66	176	0.657										
	13.0	2.20	187	0.470										
	20.0	1.31	164	0.357										
SR 3008	2.00	17.6	170	3.29	-0.018 ± 0.11	1.9 ± 0.10	0.32 ± 0.19	117 ± 21	0.19	10	0.27	380	108	14
	4.00	5.86	151	1.17										
	6.00	3.51	115	0.576										
2 PAM	0.0400	1156	200	0.964	0.11 ± 0.050	0.0075 ± 0.0008	0.017 ± 0.003	15 ± 14	6.7	61	0.11	5500	1600	205
	0.0800	431	129	0.506										
	0.100	346	55.5	n										
	0.160	216	53.6	0.203										
	0.200	175	35.4	n										
	10.0	3.50	8.13	0.122										

[a] See Table II.
[b] Calculated from [InX] and equation (9).
[c] From k_{obs} values of Table VI; $k_a = 1.61 \times 10^{-3}$ min^-1, see text.
[d] $[InX']/[EOX] = (1 - \Sigma r_i) \div (\Sigma r_{co})$ where Σr_i, is taken from Table VI.
[e] Intercept of least squares plot of data according to equation (19).
[f] Slope of least squares plot of data according to equation (19).
[g] Slope of least squares plot of data according to equation (20).
[h] Intercept of least squares plot of data according to equation (20).
[i] Calculated as shown in Table V.
[j] Calculated as shown in Table V.
[k] Calculated from equation (21).
[l] Calculated from equation (21).
[m] Calculated from equation (22).
[n] See Table VI.

These caveats notwithstanding, the kinetic constans given in Table VII do reflect some significant structure-activity relationships for the reactivation in question. Concerning the rate of dealkylation of the inhibited enzyme-oximate complex, the values of k_a' range from 0.19 to 2.4×10^{-3} min^{-1} for SR 3008, 2461, and 2462. These compare to $k_a = 1.61 \times 10^{-3}$ min^{-1} for the inhibited enzyme in the absence of reactivator and indicate no significant effect of the thiohydroximates on the rate of dealkylation. For 2-PAM, however, $k_a' = 6.7 \times 10^{-3}$ min^{-1}, suggesting that 2-PAM accelerates the aging of DFP-inhibited enzyme. Similar effects of pyridinium oximes on dealkylation of ethyl N,N-dimethylamino phosphor-amidocyanidate-inhibited AChE were reported by De Jong and Wolring.[71] The possibility that accelerated dealkylation is a general phenomenon for reactivation of phosphorylated AChE by pyridinium oximes is interesting and should be investigated further.

Insofar as reactivation is concerned, 2-PAM is characterized both by a strong affinity for the inhibited enzyme and high nucleophilicity toward phosphorus. Our values of $K_r = 0.11 \times 10^{-4}$ M and $k_r = 61 \times 10^{-3}$ min^{-1} for 2-PAM combine with a pKa value of 7.99 to give an effective bimolecular rate constant, k_{eff}, for reactivation of 1600 M^{-1} min^{-1}. By this measure 2-PAM is 200 times more effective than SR 2461 and approximately 15 times more active than SR 3008, the most effective of the SR-series thiohydroximates. It is intersting to note that for the thiohydroximates, the displacement rate constant, k_r, shows little dependence on reactivator structure, whereas values for K_r vary by a factor of 25 for the compounds examined. This observation can be used to rationalize the relative activities of the compounds. It also demonstrates how the precise determination of reactivation

kinetics for a series of related thiohydroximates could be used to "map" the inhibited enzyme, thereby providing insight into the molecular requirements for reactivation. Unfortunately, DFP proved to be a poor choice of inhibitor for these determinations because of uncertainties imposed by the rapid rate of dealkylation. A reasonable alternative to DFP would be ethyl p-nitrophenyl methylphosphonate,[73] an inhibitor that yields a phosphonylated enzyme that undergoes neither dealkylation nor spontaneous reactivation to a significant degree at 25°C.[74] Investigations along these lines are in progress and will be reported in a separate communication.

Conclusions

The SR-series thiohydroximates are moderately active reactivators
of diisopropyl phosphoryl-AChE. In this respect they should be compared
to reactivators such as TDA-5[48] and HS6[49,75] that are inferior to 2-PAM,
TMB-4, or toxogonin as reactivators, but that nevertheless are effective
in therapy of organophosphorus agent intoxication. If the thiohydroximates
combine significant activity as reactivators with low toxicity, prolonged
biological half-life, and improved tissue penetration, they should prove
to be useful antidotes. However, the toxicological and pharmacological
behavior of the thiohydroximates remains to be established.

We regard the thiohydroximates as a significant advancement in the
study of organophosphorus agent therapy not so much because *they are*
clearly superior reactivators, but because they represent a novel class
of materials and offer the promise of an extremely useful research tool
for probing the mechanisms of cholinesterase reactivation on the molecular
level. We have shown that the synthesis of thiohydroximates is at once
facile and highly flexible. Readily available starting materials can
be used to prepare a variety of hydroximoyl chlorides, each of which can,
in turn, be esterified with a series of dialkylaminoalkane thiols. This
readily allows systematic variation not only of the oxime acid dissoci-
ation constant and nucleophilicity but also of structural features in
the portion of the molecule that must interact with the anionic region
of the AChE active site. We have demonstrated that there are pronounced

structural effects on the activity of the thiohydroximates and that these can be quantitated through determination of reactivation kinetics.

The appreciation of structure-activity relationships to reactivation of inhibited-AChE has led to important advances in OP agent therapy with respect to development of pyridinium oximes. It is our hope that the thiohydroximates can be applied similarly to the design of improved therapeutics based on nonquaternary reactivators.

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Appendix

MINC-11 COMPUTER PROGRAM FOR ENZYME KINETICS

MINC ENZYME KINETICS PROGRAM (ENKIN)

The program ENKIN is designed to determine initial rates of enzyme reactions. Data are treated by the method of least squares. The program is written for the MINC-11 and controls the acquisition and manipulation of absorbance data from a Gilford 2000 spectrophotometer. Permanent storage of the data in a disc file is provided for. The program is divided into the following sections:

1. Input of identification data and operating parameters.

The main purposes for this section are to set up the disc file with identification and a label and to acquire the input parameters "SCALE FACTOR" and "DATA POINTS PER CUVETTE." The disc file setup requires the following responses from the operator.

a. NAME. The first line of the disc file includes the operator's name. There is no restriction on the type of characters except that they must fit on one line (80 character maximum).

b. IDENTIFIER. As part of the identification of the data for the disc storage file, the time and data are automatically stored after the operator's name. In addition, a further experiment IDENTIFIER is provided for. It consists of up to 80 characters of any type that the operator chooses to aid in the identification of the particular experiment.

c. FILE NAME. The file name is the name that the MINC uses to identify the data file. It appears in the directory. The FILE NAME may have up to six alphanumeric characters, but the first character must be

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a letter. The program assigns the file to the left-hand disc (SYO:) and assigns the extension .DAT to the file unless otherwise specified. The complete file name is thus SYO:xxxxxx.DAT. The right-hand disc (SY1:) can be specified, and the extension may take any other three-letter value that the operator includes as part of FILE NAME.

The next operation is acquisition of two operating parameters.

d. SCALE FACTOR. The program ENKIN takes electrical signals from the recorder input of the Gilford 2000 spectrophotometer. Whatever value of absorbance that gives a full-scale reading on the recorder gives a value of 1.000 to the program. Thus if full scale on the recorder is adjusted to be 0 to 1.0 absorbance units, the SCALE FACTOR should be 1.0. If full scale is 0.0 to 0.1, the SCALE FACTOR is 0.1. The SCALE FACTOR can be used to convert absorbance into concentration units. To accomplish this, the operator must enter the concentration that corresponds to full scale on the recorder.

e. DATA POINTS PER CUVETTE. The program provides for up to 100 data points for each cuvette. The actual number of points must be specified by the operator. The program examines each cuvette, records data if available, and increments a counter if the data were recorded. This process is repeated until one of the counters has the same value specified in DATA POINTS PER CUVETTE, at which point the program ceases to take data. The actual number of data points is recorded for each cuvette.

2. Accumulation of data and updating of display.

For the accumulation of data, two interfaces are used. The first is the MINC preamplifier channel A. Its input must be connected in parallel with the 50-mV input to the chart recorder. The preamplifier is placed

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on the left side of the MINC analog-to-digital converter (ADC), and the
preamplifier output is thus routed to channel 8 of the ADC.

The second interface consists of four photodarlington pilot light
detectors. The pilot light detectors require +5V and ground. The detectors
for pilot lights 1, 2, 3, and 4 are connected to input channels 4, 5, 6,
and 7, respectively, of the ADC. A signal of greater than 1 volt is
detected if the pilot light is on. (The signal is actually a ripple
voltage, the shape of which depends on the particular light source.)

Data from the first interface are acquired using a subroutine
which returns the sum of 20 samples of the ADC. Since full-scale input
to the recorder is 50 mV, the sum of 20 samples of a full-scale reading
is 1.0 V. There is thus no adjustment required in order to represent
fraction of full scale. The time (in minutes) is evaluated after the first
ten samples and before the second ten samples are summed.

Data from the second interface are also acquired by a subroutine.
The subroutine samples each pilot light consecutively. If a pilot light
is found on, the lamp indicator variable is set appropriately and control
is returned to the main program. If no pilot light is found on after
the last one is checked, the checking procedure is started over at pilot
light No. 1.

A brief flow chart of the section 2 program operation is shown
in Figure A-1. Cuvette number has the symbol C. Q and Q9 are temporary
values of C. V2 is a temporary value of absorbance, and Y is either
absorbance or concentration, depending on the value and units of the SCALE
FACTOR. The symbol X is used to represent time in those calculations that
involve summations. Similarly, N is used to indicate point count. The

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following formulas are used to calculate slope, intercept, correlation

coefficient (R), and standard deviations of slope and intercept [S (slope)

and S (intercept)].

$$\text{slope} = \frac{N\Sigma XY - \Sigma X\Sigma Y}{N\Sigma X^2 - (\Sigma X)^2}$$

$$\text{intercept} = \frac{\Sigma X^2\Sigma Y - \Sigma X\Sigma XY}{N\Sigma X^2 - (\Sigma X)^2}$$

$$R = \frac{N\Sigma XY - \Sigma X\Sigma Y}{(N\Sigma X^2 - (\Sigma X)^2)^{1/2}(N\Sigma Y^2 - (\Sigma Y)^2)^{1/2}}$$

$$S \text{ (slope)} = \left(\frac{TN}{N\Sigma X^2 - (\Sigma X)^2}\right)^{1/2}$$

$$S \text{ (intercept)} = \left(\frac{T\Sigma X^2}{N\Sigma X^2 - (\Sigma X)^2}\right)$$

where

$$T = \frac{\Sigma Y^2 - \dfrac{(\Sigma Y)^2\Sigma X^2 - 2\Sigma XY\Sigma X\Sigma Y + N(\Sigma XY)^2}{N\Sigma X^2 - (\Sigma X)^2}}{N - 2}$$

3. Data Storage.

After NAME, time, date, IDENTIFIER, and SCALE FACTOR, the kinetic

data are stored. For each cuvette, the least squares slope and intercept,

the correlation coefficient, and the number of data points are stored.

Next, the standard deviations of the slope and intercept are stored for

each cuvette. This is followed by a list of times (in minutes) and ab-

sorbances (or concentrations depending on the SCALE FACTOR) for each cuvette.

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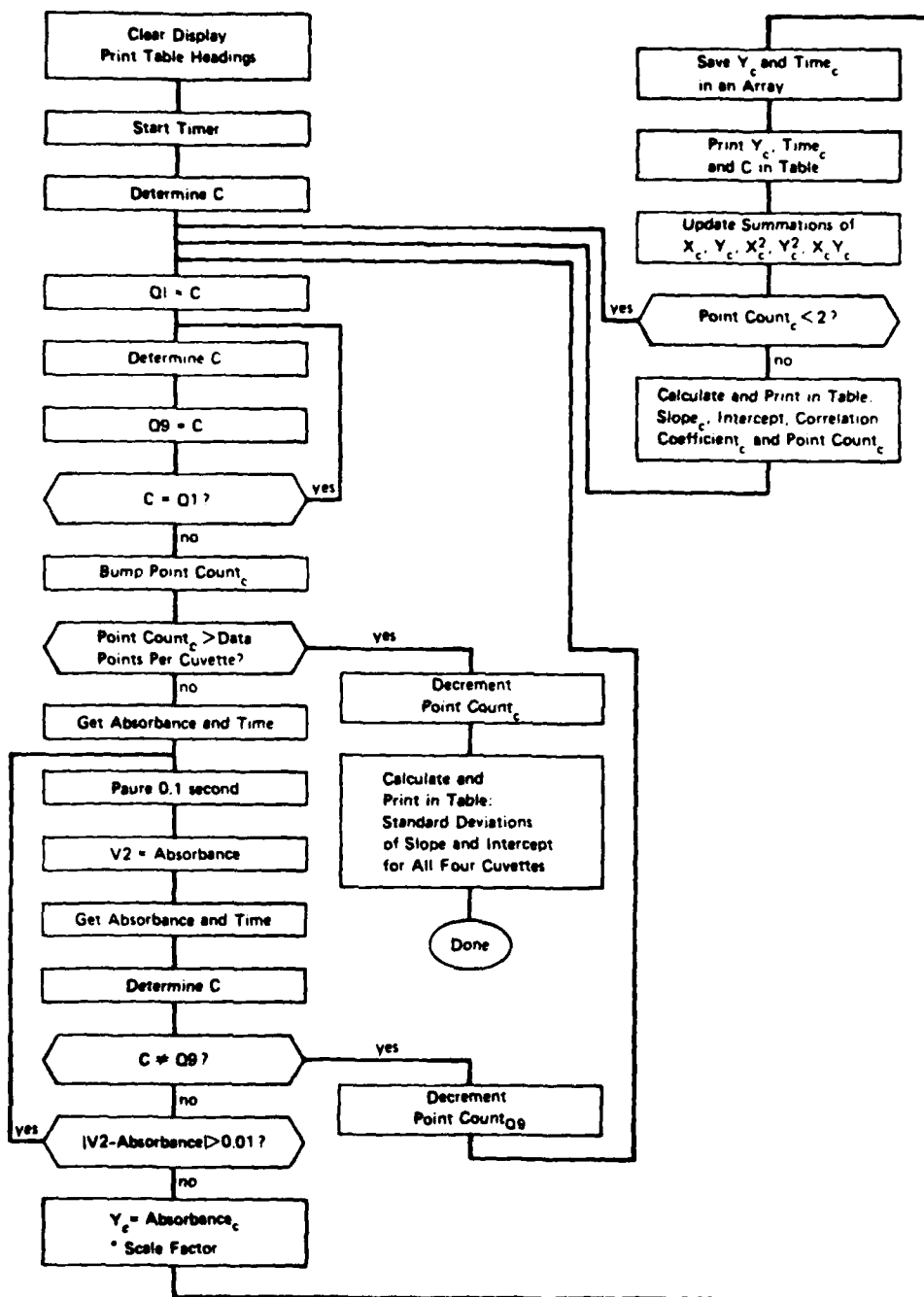
JA-1043-8

FIGURE A1   FLOW CHART OF SECTION 2 OF ENKIN

The operator can examine these data using the command TYPE followed by
the FILE NAME. Hard copy of the data files is not possible unless a line
printer is available. For printed copies, the floppy disceltes can be
transported to any LSI-11 computer that has a line printer and RX02 disc
drive.

4. Graphics.

Data may be displayed on the terminal using MINC graphics routines.
To skip this feature, the operator must enter a zero. Otherwise the cu-
vette number must be entered. All four cuvettes can be examined by se-
quentially typing the appropriate cuvette numbers.

```
10 REM - ENKIN is a program for determining initial rates of enzyme
20 REM - catalyzed reactions.  It takes absorbance data from a Gilford
30 REM - 2000 spectrophotometer and treats it by the method of least
40 REM - squares.
50 REM
60 REM - Section 1  Input of ID data and operating procedures
70 DIM X(3),Y(3),X2(3),Y2(3),X9(3),N(3)
80 DIM A(3),B(3),R(3),S1(3),S2(3)
90 DIM P(3,100),Q(3,100)
100 PRINT 'ENTER YOUR NAME PLEASE'
110 LINPUT N$
120 PRINT 'ENTER IDENTIFIER FOR THIS EXPERIMENT'
130 LINPUT E$
140 PRINT 'ENTER FILE NAME FOR THE DATA'
150 LINPUT F$
160 OPEN F$ FOR OUTPUT AS FILE #1
170 PRINT #1,N$,CLK$,DAT$
180 PRINT #1,E$
190 PRINT 'ENTER NUMBER OF DATA POINTS PER CUVETTE'
200 INPUT N1
210 IF N1>100 THEN PRINT '100 IS THE MAXIMUM - TRY AGAIN' \ GO TO 200
220 PRINT 'ENTER SCALE FACTOR'
230 INPUT F5
240 PRINT #1,'Scale Factor ';F5
250 REM
260 REM - Section 2  Accumulation of data and updating of display
270 DISPLAY_CLEAR
280 MOVE_CURSOR(1,1)
290 PRINT 'Kinetic Parameters (in minutes) for Cuvettes 1 to 4'
300 PRINT 'Slope','Intercept','R','N'
310 MOVE_CURSOR(10,1)
320 PRINT 'Cuvette','Time','Absorbance (or concentration)'
330 START_TIME('CHZ')
340 REM
350 REM - C is cuvette number, X1 is time, V is absorbance (Orconc.)
360 GOSUB 1110
370 Q1=C
380 GOSUB 1110
390 Q9=C
400 IF C=Q1 THEN  GO TO 380
410 N(C)=N(C)+1
420 IF N(C)>N1 GO TO 700
430 GOSUB 1230
440 PAUSE(.1)
450 V2=V
460 GOSUB 1230
470 GOSUB 1110
480 IF Q9<>C THEN N(Q9)=N(Q9)-1 \ GO TO 370
490 IF ABS(V-V2)>.01 THEN  GO TO 440
500 V=V*F5
510 P(C,N(C))=V
520 Q(C,N(C))=X1
530 MOVE_CURSOR(11,1)
540 PRINT C+1,X1,V,'           '
550 X(C)=X(C)+X1
560 Y(C)=Y(C)+V
570 X2(C)=X2(C)+X1*X1
580 Y2(C)=Y2(C)+V*V
590 X9(C)=X9(C)+X1*V
600 IF N(C)=1 GO TO 370
```

```
610 D=N(C)*X2(C)-X(C)*X(C)
620 A(C)=(X2(C)*Y(C)-X(C)*X9(C))/D
630 B(C)=(N(C)*X9(C)-X(C)*Y(C))/D
640 R(C)=(N(C)*X9(C)-X(C)*Y(C))/SQR(D*(N(C)*Y2(C)-Y(C)*Y(C)))
650 MOVE_CURSOR(4+C,1)
660 PRINT B(C),A(C),R(C),N(C)
670 GO TO 370
680 REM
690 REM - Data gathering loop ends here
700 N(C)=N(C)-1
710 MOVE_CURSOR(13,1)
720 PRINT 'S(slope)','S(intercept)'
730 FOR L=0 TO 3
740 T=(Y(L)↑2*X2(L)-2*X9(L)*X(L)*Y(L)+N(L)*X9(L)↑2)/(N(L)*X2(L)-X(L)↑2)
750 T=(Y2(L)-T)/(N(L)-2)
760 S1(L)=SQR(T*X2(L)/(N(L)*X2(L)-X(L)↑2))
770 S2(L)=SQR(T*N(L)/(N(L)*X2(L)-X(L)↑2))
780 PRINT S2(L),S1(L)
790 NEXT L
800 REM
810 REM - Section 3  Data storage
820 PRINT #1,'Slope','Intercept','R','N'
830 FOR L=0 TO 3
840 PRINT #1,B(L),A(L),R(L),N(L)
850 NEXT L
860 PRINT #1,'S(slope)','S(intercept)'
870 FOR L=0 TO 3
880 PRINT #1,S2(L),S1(L)
890 NEXT L
900 FOR L=0 TO 3
910 PRINT #1,'Time','Absorbance','Cuvette ';L+1
920 PRINT #1,,'(or concentration)'
930 FOR C=1 TO N(L)
940 PRINT #1,Q(L,C),P(L,C)
950 NEXT C
960 NEXT L
970 CLOSE #1
980 REM
990 REM - Section 4  Graphics
1000 PRINT 'ENTER CUVETTE #      ENTER ZERO TO SKIP GRAPHIC ROUTINES'
1010 INPUT N4
1020 IF N4=0 THEN DISPLAY_CLEAR \ GO TO 1350
1030 IF N4>4 THEN PRINT 'TYPE 1, 2, 3, OR 4' \ GO TO 1000
1040 DISPLAY_CLEAR
1050 GRAPH("LINES",N(N4-1),Q(N4-1,1),P(N4-1,1))
1060 PRINT 'DATA FROM CUVETTE ';N4
1070 GO TO 1000
1080 GO TO 1350
1090 REM
1100 REM - Subroutine determines position of cuvette
1110 H=1
1120 AIN(,W,,,4,)
1130 IF W>H THEN C=0 \ RETURN
1140 AIN(,W,,,5,)
1150 IF W>H THEN C=1 \ RETURN
1160 AIN(,W,,,6,)
1170 IF W>H THEN C=2 \ RETURN
1180 AIN(,W,,,7,)
1190 IF W>H THEN C=3 \ RETURN
1200 GO TO 1120
```

```
1210 REM
1220 REM - Subroutine determines absorbance and time
1230 V=0
1240 FOR L=1 TO 10
1250 AIN( ,V1,,,8, )
1260 V=V+V1
1270 NEXT L
1280 GET_TIME( X1 )
1290 X1=X1/60
1300 FOR L=1 TO 10
1310 AIN( ,V1,,,8, )
1320 V=V+V1
1330 NEXT L
1340 RETURN
1350 END
```

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